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PROVISIONAL APPLICATION COVER SHEET

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TITLE OF THE INVENTION (280 characters max)

MONOCLONAL ANTIBODY BASED BIOMARKER DISCOVERY AND DEVELOPMENT PLATFORM

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Monoclonal Antibody Based Biomarker Discovery and Development Platform

Abstract

A novel Platform for monoclonal antibody based biomarker discovery and development is disclosed herein. The Platform of the invention provides for the integration of systems biology, hybridoma screening and nano-volume integrated mass spectrometry to achieve a robust screening system that cuts 4-6 years off of the classical biomarker R&D process. A key feature of this novel Platform system is its ability to produce disease specific biomarkers.

Background

Biomarkers are surrogate clinical measures for analyzing drug safety and early efficacy in testing lead compounds. They are used in clinical assays that are carried out to assist in the management of disease. Disease management benefits from diagnosis, staging, stratification and measures of progression and prognosis, but, most importantly, from early measures and/or predictors of drug efficacy or toxicity. Different types of clinical biomarkers can be used in all these areas. The pharmaceutical industry is interested in biomarker discovery because of two main reasons.

First, the increasing rate of drug candidate attrition has reached levels where the cost effectiveness of drug discovery and development becomes questionable (if profit margins are kept at current levels). The root causes of drug candidate attrition have been identified as resulting from a poor understanding of the mechanism of action of the candidate and from poor pharmacological validation and translation of cellular and animal model-based results to the clinic. The use of biomarkers can bridge the gap between cellular and animal models and

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human clinical conditions, and new biomarkers, such as HER2, are likely to be relevant to drug mechanisms of action as predictors of drug efficacy. Another major cause of attrition is the individuality of drug toxicity reactions. Identification of individuals with idiosyncratic and other unexpected responses will save lives and money and will allow the introduction of safer drugs. Recent examples of efficient genetic biomarkers of this type have been reported.

Secondly, the high cost of clinical trials for candidate drugs for slowly progressing chronic diseases is prohibitive. Alzheimer disease, type II diabetes, cardiovascular diseases, rheumatoid arthritis, osteoarthritis and chronic obstructive pulmonary disease represent a major fraction of health care costs and contribute significantly to the direct cause of death. The market and the public's need are tremendous in these disease areas, which beg for effective mechanism based drugs. Yet, the slow progressive nature of these diseases poses a currently impenetrable problem. The minimal measurable improvement (15-30%) in disease symptoms occurs over such a long period of time that it is impractical and too expensive to test potential therapies in clinical trial settings. The expectation for disease progression specific biomarkers is that they will predict improvement earlier than such improvement actually occurs, thus providing a useful tool to measure and predict the efficacy of novel drugs in shorter and less expensive clinical trials. Consequently, the first tests of the new biomarkers will have to be carried out in long Phase II/III clinical trials in close collaboration with pharmaceutical companies.

Description of the invention

Described herein is a novel and integrated, high throughput, disease-specific mAb based biomarker discovery and development platform.

To eliminate discovery process bottlenecks and to keep biomarker discovery methods global until the last validation and subsequent development steps,

careful considerations have to be made with regards to the integration process of divergent but essential genomics, genetics, proteomics, metabolomics and bioinformatics technologies and the information derived from these. Without a specific integration strategy, success will suffer from the inability to deliver appropriate clinical assay candidates on the larger scale or the lack of robustness in addressing the entire genome, proteome or metabolome. Biomarker discovery processes built on single and dominant discovery technologies, e.g., systems biology, mass spectrometry or mAb-based approaches, are inefficient as the outcome of these strategies is not built on global analysis and/or the results require an additional, lengthy and inefficient step: translation to clinical assay candidates, which then feed into the biomarker development process. The harmonized technology and global information integration strategies described herein as part of this invention provide global analysis and fast and easily adaptable results, with smooth progression to biomarker development in the form of clinical assay candidates, as opposed to candidate genes or expression patterns (the result of single technology driven approaches).

The desired end outcome of a biomarker discovery process is a highly specific and sensitive clinical assay. In practice, monoclonal antibody (mAb), quantitative PCR (qPCR) and single nucleotide polymorphism (SNP) genotyping based technologies are pertinent to all genes for the measurement and prediction of disease progression, disease treatment and experimental condition caused effects. Monoclonal antibody, qPCR and SNP genotyping based single and multiplexed assays are easily applicable to routine clinical laboratories and readily amenable for point of care device development. Although the Platform according to the invention generates qPCR and SNP based clinical assay candidates, the focus of the invention described herein is on large scale discovery and production of mAb based, disease specific clinical assay candidates. The actual and precise measurement of proteins, protein modification and metabolites is more relevant to disease diagnosis, follow-up and

experimental conditions than the measurement of mRNA expression levels or statistical probabilities associated with particular genotypes.

In order to ensure the generation of mAb based clinical assay candidates as the outcome of the global (genome-, proteome- and metabolome-wide) biomarker discovery and development process, the Platform according to the invention integrates three major technology components, Systems Biology, Hybridoma Screening and Nano-Volume Integrated Mass Spectrometry (NVIMS.)

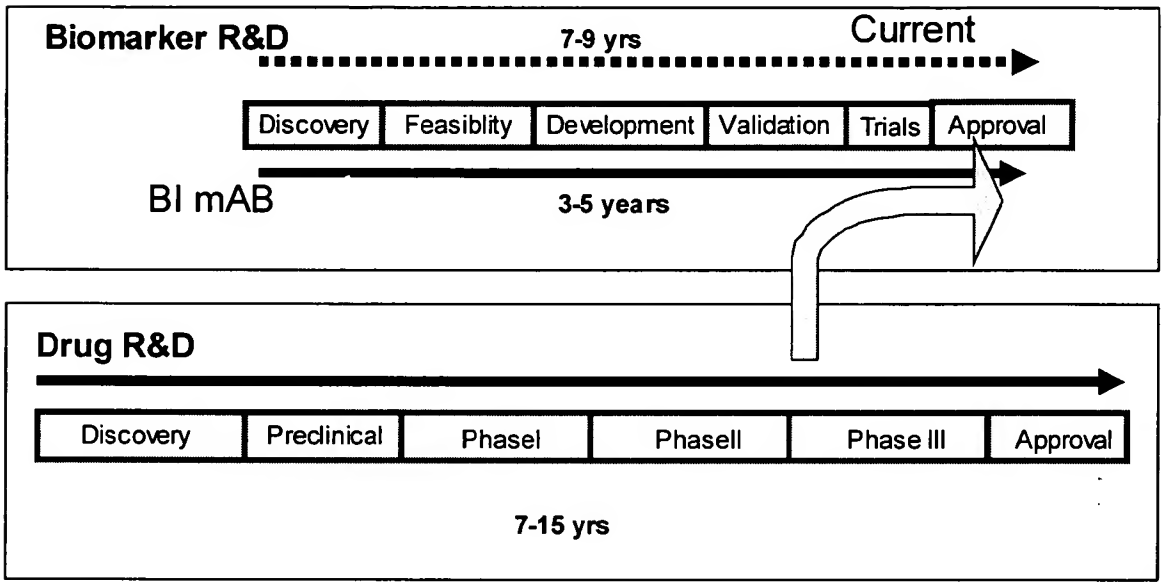

Fig. 1

As illustrated in Fig. 1, the classical biomarker R&D process takes about 7-9 years from discovery to approval. The discovery phase of the process is relatively short (1-3 years). However, the clinical feasibility phase, assay development, clinical validation, trial test and approval phases take an additional 6-8 years. The mAb based Platform according to the invention, because it uses clinical samples from a patient pool similar or identical to patients in clinical trials, achieves the clinical development and biomarker discovery phase simultaneously and saves time (4-6 years shorter). Thus, this mAb based strategy is significantly faster than the classical biomarker R&D process. Furthermore, this Platform mAb

strategy can be applied at a global level, keeping the advantages of Systems Biology.

The Platform outline is shown in Fig. 2.

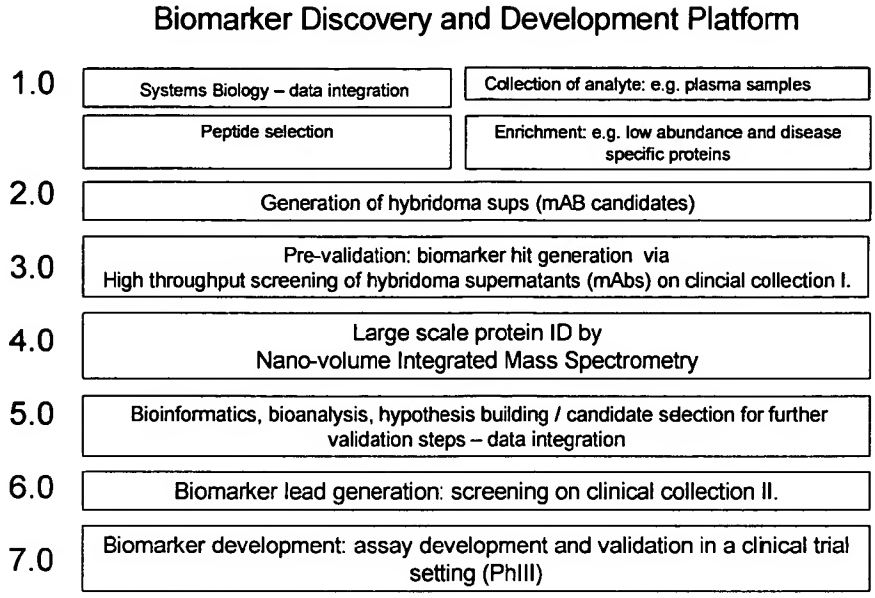

Fig. 2

The Platform according to the invention is designed for the discovery of early disease-specific clinical efficacy biomarkers, with the primary goals being (i) to reduce clinical trial length of candidate therapies for chronic diseases and (ii) to predict and follow treatment efficacy of marketed drugs. However, the Platform, albeit with minor modifications, is also applicable to the analysis of non-human samples and problems outside of disease diagnosis and treatment (e.g., ecological and biohazard applications). Depending on the intended application, the complex analyte (see details below) chosen as a source of potential biomarkers might vary.

The Platform comprises the following process steps.

1. Generation of appropriate antigens using both a Systems Biology approach and a Collections approach

Systems Biology, as used herein and as described in more detail in Example I, means the collection and analysis of biologically relevant and global genomic, genetic, proteomic and metabolomic data from experiments and from the literature and systematically mining these data to find biomarker candidates.

The potential antigenic regions from candidate protein or polypeptide biomarkers identified using the Systems Biology approach are mapped for each of the candidates using antigenicity prediction algorithms, e.g., DNAStar. The peptide regions chosen are those showing the greatest probability for surface exposure that do not contain cysteine, as an N-terminal cysteine is included on each peptide to facilitate coupling to Keyhole Limpet Hemocyanin (KLH), a common carrier protein used for peptide immunization. Multiple 12mer peptides are designed for each protein.

Although it is not the focus of this invention, a Systems Biology strategy will also generate biomarker candidate genes with differential mRNA expression levels and/or genetic associations. These are translated to clinical assay candidates by specific qPCR and genetic polymorphism (SNP) assay design and validation. These assays could be validated as independent biomarkers or as biomarkers combined with mAb based biomarkers. It is likely that these cross-platform, multiplexed biomarkers (qPCR/genetic/protein) will have important value.

Analyte collection, the second method of generating antigens, achieves the generation of a small set of analyte samples that represent one or multiple diseases or experimental conditions with one or multiple sets of controls. The conditions are chosen to include clinical symptoms and/or disease stages that will have to be predicted by the newly discovered biomarkers before the actual appearance of the disease or condition outcome. The number of individual samples might not exceed 50 in any of the groups. In order to speed the biomarker discovery process, partial collections could be used at this stage.

Sample collection is driven by clinical data and their interpretation, based on the best available medical practice. The resulting inclusion and exclusion criteria are set with physician experts and approved by regulatory bodies.

Enrichment of the collected sample low abundance and/or disease-specific proteins can be performed based on any desired and suitable biological or physicochemical characteristics of the targeted complex analyte samples (e.g., concentration, mass). A specific two step immuno-affinity absorption strategy (described in Example II) involves (i) depletion of the most abundant proteins (e.g., albumin and immunoglobulins from plasma) from the analyte pool (since these proteins are not expected to have biomarker value yet might represent >90% of the total protein) and (ii) removal of proteins reacting with polyclonal antisera that are generated to one set of analytes in the pool (e.g., control). The analyte is enriched for proteins that were originally present only at low concentration (e.g., <5%) and proteins that might be present only in one set of the analytes. For enrichment purposes, complex analyte samples can be pooled.

Other enrichment strategies using ligand affinity chromatography or separation technologies that enrich proteins based on their size, charge or binding characteristics to other proteins, etc., can also be used.

2. Generation of hybridoma supernatants

This step involves well-established technologies aimed at the generation of mAbs to individual components of complex antigen mixes. The technical steps are as follows: (1) immunization of mice with complex antigens such KLH coupled peptide mix or enriched analyte samples; (2) hybridoma fusion; (3) culturing of fused hybridomas under limiting dilution conditions in microtitre wells; (4) harvesting of hybridoma supernatants; and (5) freezing and storage of hybridoma cells.

3. Pre-validation: Biomarker hit generation via high-throughput screening of hybridoma supernatants (mAbs) on a first Clinical Collection

A well-characterized representative group of individual analyte samples, e.g., 50 plasma samples from a specific disease or disease condition and 50 samples from appropriate control subjects is selected. A high throughput assay format is then developed to screen the analyte samples with the mAbs present in hybridoma supernatants.

In the example presented, a capture micro-ELISA assay is shown, which (i) captures the Ig from the hybridoma supernatant in the first step; (ii) subsequently, biotinylated candidate biomarker, prepared from pooled non-enriched or pooled enriched analyte, is used to detect anti-biomarker reaction; and (iii) in the third step, non-enriched individual analyte samples are titrated to compete with fixed amounts of candidate biomarker.

Screening assays built on other principles than ELISA can be deployed (e.g., antibody microarrays, high throughput screening based on MALDI/MS, multi-channel capillary electrophoresis, etc.).

ELISA or microarray data are evaluated by published methods and public (e.g., Spotfire) or software according to the invention. The goal of the data analysis is the selection of hybridoma supernatants that show the best collection with an important clinical parameter and are specific to one of the analyte groups.

4. Protein ID by Nano-Volume Integrated Mass Spectrometry (NVIMS)

In this step, the small quantity of antibody present in 5-200µl hybridoma supernatant is captured, and an advanced high throughput mass spectrometry

based technology is used to identify the antigen to which it binds. Various methods useful in carrying out this step are described in Example III.

Advances in proteomics technologies allow affinity purification of antibodies and the antigen that is recognized by them on the nano/pico-scale; ng/pg quantity of Ig will be purified from sub ml amounts of hybridoma supernatants, and quasi equimolar quantities of the antigen will also be purified. Nano-volume scale HPLC / CE columns in boundless or special microfluidics devices or nanowell plate devices will be coupled to high sensitivity FT-MS to achieve high throughput protein ID (Fig. 3). If the throughput of the technology allows it, mAb libraries will be built in the form of hybridoma supernatants and frozen hybridoma cells.

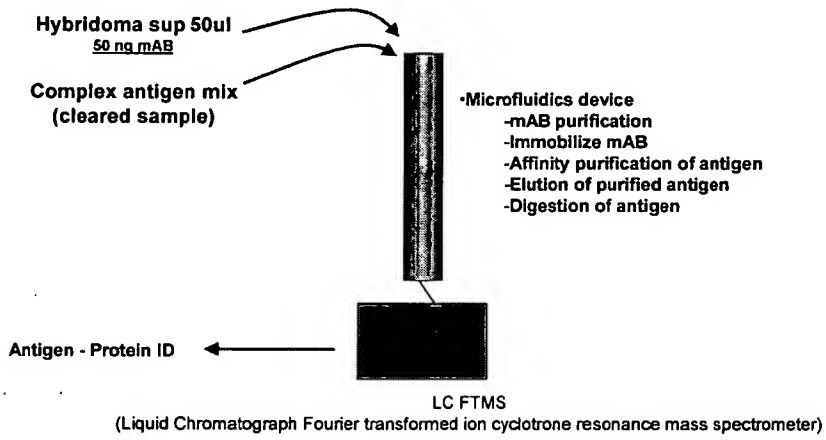

Fig. 3

5. Bioinformatics, bioanalysis, hypothesis building / candidate selection for further validation steps – data integration

In this step, data obtained from the Platform are contrasted to current medical knowledge and experimental data available. The Systems Biology strategy is deployed again for electronic prioritization of candidate biomarker hits. Prioritization concepts are driven by biomarker need (e.g., response to treatment, disease progression, disease improvement, toxicity, etc.) and available data on specificity and sensitivity of the mAb based assays. A critical next element of the

prioritization process is bioanalyis, which examines top candidates one by one. This is the first non-electronic data analysis step. If done in parallel with steps 3 and 4, the analysis will select candidates for which screening assay can be repeated in a more precise format (e.g., not pooled) for the generation of higher confidence level (strict statistical term) mAb based candidates.

6. Biomarker lead generation: screening on an extended clinical collection

In order to progress to biomarker lead and to generate data for the early biomarker development steps, a second discovery level validation step is deployed, which is performed on a larger analyte collection, typically 250 individual samples (patients or subjects) in each group. Exclusion and inclusion criteria are designed by clinical need and by epidemiologic data (if available). As for the first clinical collection, each subject is requested to provide samples for DNA testing.

In order to perform the screening on the extended clinical collection, a robust, mAb based, research level clinical assay is needed. This assay is developed as a single ELISA-like or other single mAb based assay or as an assay multiplexed from various mAbs or from various platforms (qPCR, SNP genotyping). The steps involved are the following.

(i) Hybridoma cloning mAb production (well established published technologies): Selected frozen hybridoma cells are thawed and cloned. To follow the cloning procedure and hybridoma verification purposes, the assay used in steps 3 and 4 are repeated until the secure establishment of at least three hybridoma cell lines that secrete sufficient quantity of mAb has been obtained. After three cycles of repeated cloning, isotype determination and freezing of multiple samples, milligram quantities of mAbs are produced that are quality controlled by the procedure described in step 4 and/or by classical immunoaffinity chromatography followed by Edman protein sequencing.

Individual hybrid clones are subjected to a PCR mediated IgH and L chain variable region cloning procedure.

In contrast to screening of the first clinical collection, the analyte samples are not pooled. The research level clinical assay candidate, a single mAb based or multiplexed assay, is used to screen each individual clinical sample. In summary, the process steps are sample collection, screening and primary data analysis.

Genetic association of candidate biomarkers with disease or biological phenotype provides the best initial and hypothesis-free experimental evidence for relevance. With sufficiently large clinical collections, genetic association studies such as are described below, can provide this powerful evidence. The process steps are (i) database mining to collect all available genetic polymorphism information on selected candidate genes; (ii) if necessary, a SNP discovery study, with subsequent selection of a small set of informative haplotype defining SNPs; (iii) genetic screening; (iv) statistical analysis of candidate gene polymorphisms association with relevant biological or disease phenotypes; and (v) search for supporting biological or experimental evidence (e.g., the role of a coding polymorphism changing biology relevant receptor phenotype).

A third cycle of System Biology / bioanalysis is performed with the involvement of biomarker and clinical diagnostics assay developer specialists. The final list of biomarkers is selected for development.

7. Biomarker development: assay development and validation in clinical trial setting

At this point, marketable assay candidates are developed. These candidates could be simple ELISAs, multiplexed assays os point of care devices.

Additionally, phase II and phase III clinical assays are developed. Specifically, the question of whether the biomarker assay predicts disease progress or treatment outcome earlier than the actual occurance will be tested.

Definitions and Examples

Complex analytes as immunogens (defintion):

The Platform ensures fast and high throughput generation of monoclonal antibodies of high specificity to individual components of complex analytes. Complex analytes here mean:

(i) Naturally occurring complex protein mixtures

(ii) Complex protein mixtures of natural origin enriched for components with specific features, like proteins specific for a disease or a clinically, pathologically or physiologically defined condition, or proteins sharing physicochemical properties (e.g., charge, mass or abundance)

(iii) Artificial mixtures of purified or recombinant protein mixes, and artificial mixtures of synthetic peptides

(iv) Naturally occurring mixes of organic metabolites

(v) Artificial mixes of naturally occurring but enriched or purified organic compounds

(vi) Mixes of compounds of synthetic origin

Complex analytes of biological origin represent human and animal serum or plasma; proteins of urine, tear, sputum, inflammatory exudates (e.g., synovial fluid, cysts, bursas, etc.); any normal or pathological bodily fluid or excretion, including faeces, tissue extracts of normal and pathological tissues (e.g., malignant and benign tumors, cancerous tissue, biopsy material of normal and pathological tissues, e.g., biopsies of the colon, breast, liver, kidney). Complex analyte mixtures also include extracts and lysates of bacteria; bacterial, fungal

and higher organism composed ecosyststems; and extracts or condensates of soil, clouds or air (e.g., exhaled air).

EXAMPLE I

Systems Biology (Parallel Biology™ data integration process)

The Systems Biology approach used in the novel Platform of the invention is an integrated analysis process of assembling and extracting the essence from divergent biological information (e.g., gene expression analysis, proteomics studies, published information, genetic association). Our novel electronic integration process is named Parallel Biology. It starts with the generation of primary candidates that qualify because they are: (i) present in, (ii) up or down regulated by, (iii) chemically modified in or (iv) represent genetic risk to the pathological, physiological or experimental (e.g., drug treatment) process under investigation. In the subsequent steps, attributes for each primary candidate are generated via the collection of additional types of information. These attributes are then expressed in either (i) binary, (ii) normalized numerical or (iii) other computable formats. Finally, values are multiplied by specific weighting factors that are applied empirically based on concepts that drive the prioritization strategy (e.g., ability to be converted into a drug, disease relevance, etc.),. The computed sum of weighted attribute values is used for sorting candidate biomarkers.

The final list of candidates undergoes a manual bioanalysis process that evaluates the rationale for having each given candidate on the list one by one and establishes the final priority list. Peptide sequences from candidate biomarkers on this list are selected based on immunogenicity criteria and other criteria such as those that take the need for coupling to a carrier and synthesis conditions into account.

EXAMPLE II

Enrichment for low abundance and/or disease process specific proteins

The Platform allows the rapid generation of monoclonal antibodies directed toward medium to low abundance proteins representing < 10% of total complex protein mix (weight/weight). This is an important advance as complex protein mix, like plasma, contains many high abundance proteins with a dynamic range of 10^{12}, and, thus, it is necessary to deplete the abundant proteins before antibodies can be generated to the low (abundancy or level) proteins of diagnostic interest and biomarker potential.

One approach is to remove abundant proteins using antibodies or chemical ligands that have specific affinity to individual high-level proteins. In this manner, using prior art methods, up to 16 abundant proteins have been depleted from plasma (Large Scale Biology), and this approach has achieved a 10 fold enrichment of the mixture for low level proteins. This approach suffers from drawbacks, however, in that it takes considerable time to generate the specific ligands to the abundant proteins. Furthermore, there are many other high abundance proteins; so, to achieve a complete removal of abundant proteins, one would have to generate an additional 30+ antibodies and/or ligands.

The method described in this invention uses a second affinity chromatography depletion step that is mediated by a polyclonal antibody prepared against the complex analyte mix, e.g., serum or plasma proteins. This two step approach, initial depletion steps using targeted ligands followed by a polyclonal antibody column, achieves a much more complete depletion of abundant proteins. The depletion process is monitored by, e.g., 1D or 2D gel electrophoresis and LC/MS analysis.

A complex protein mix of human origin is collected from groups of subjects that are identified, e.g., by clinical tests. These groups may represent, e.g., patients

at various stages of disease vs. control subjects, or subjects exposed to drugs or environmental factors vs. those who have not been exposed.

Pools of samples are prepared by mixing equal volumes of samples, e.g., plasma or serum from the individual patients. Individual pools are subjected to the two-step affinity chromatography procedure described above. The first step uses column chromatography against immobilized antibodies or ligands to a few of the most abundant proteins in the pool. The flow through from this column, the "first cleared mix," is collected and subjected to the second step of separation.

In the second affinity chromatography step, the "first cleared mix" is loaded onto a column that contains immobilized polyclonal antibody that has been generated against one of the specific pools that are being compared or against one that contains a mix of 20-500 monoclonal antibodies to specific components of the complex protein mix. The flow through of this column, "second cleared mix," is collected.

Analysis of this second cleared mix shows that the analyte population has been enriched more than 20-fold for proteins that were less abundant in the original sample.

EXAMPLE III

Large scale protein (antigen) purification and identification

This technology builds an industrial scale process based on a recent publication demonstrating the use of MS for hybridoma characterization. This improved industrial scale process couples different devices that allow high throughput manipulations, e.g., microfluidics chips, nanowells] and/or individual or bundled capillaries to sensitive mass spectrometer(s) (e.g., FT-MS). The process of the

invention requires significantly less mAb for antigen identification than prior art processes due to miniaturization of the analytical device.

In the process described herein, capillaries, nanowells and/or microchips are sequentially arranged as functional units/surfaces that:

(i) Bind and concentrate 1-100ng mAb specifically via the Fc portion and allow the rest of the hybridoma supernatant to exit the system.

(ii) Allow micro/nano-scale affinity binding and elution of individual analyte species to/from the mAb(s) present in individual hybridoma supernatants and, thus, allow elution and concentration of affinity purified "quasi" homogeneous analyte species from complex analyte protein mixtures.

(iii) Digest the concentrated homogeneous analyte species with appropriate proteolytic enzyme(s) (e.g., trypsin) for subsequent MS analysis.

(iv) Allow easy processing of the digested analyte for loading onto a mass spectrometer.

Samples at this stage are transited to a specific coupling and loading unit that injects the sample into the mass spectrometer for analysis. In this way, the sequential identification of each individual fragment from a digested affinity purified analyte species and the identification of the mass of the fragments permits high fidelity protein ID assignment with the use of current empiric and predicted protein data sets.

This industrial scale parallel instrument will be built as described below:

Generation of bioactive solid surfaces through immobilization of antibodies is important for biomarker discovery and screening. The solid-phase environment

provides sufficient bioactivity, stability and reproducibility without a high background or loss of antigens,. The progress in microfabrication technologies and the trend towards the creation of integrated biodevices imposes a new and major constrain on immobilization techniques: the requirement for highly defined space-programming of the immobilization of biomolecules. In this process, anti-mouse IgG heavy chain Ab or Protein G will be bonded, e.g., onto the silica surfaces of microbore capillaries or microfluidic channels or the siloxane surfaces of nanowells to form highly controlled affinity surfaces. These will be used in high throughput screening (HTS) processes with no diffusion limitation.

There are two possible approaches to address this task: a flexible "lego-like" approach using pieces of microbore capillary columns connected to each other via relevant valve structures or an integrated monolithic approach by microfabrication. In both instances, there are three major parts of the assembly: (i) an immunoaffinity trapping chamber connected to (ii) a digestion chamber that is connected to (iii) a high-resolution mass spectrometer. If necessary, a separation column can be inserted between the immunoaffinity trapping chamber and the digestion chamber. The inner wall of the immunoaffinity trapping chamber is covered by covalently attached anti-mouse IgG heavy chain Ab or Protein G. In order to increase reaction surface, beads can be used in microcapillaries or microfabricated poles can be used in microfluidic devices.

Referring to Fig. 4, the background in the hybridoma supernatant is assessed by flushing the supernatant through the immunoaffinity trapping chamber to saturate the affinity surface with IgG (1->AB->2), washing the trapped IgG's with phosphate buffered saline (PBS) (1->AB->2) and elution of the IgG's with an acidic buffer system into the digestion chamber (D). The pH is adjusted through outlet #2 during the transfer (mixing). In a similar manner to the covalent coating of the immunoaffinity trapping chamber, the inner wall of the digestion chamber is covalently covered by an appropriate enzyme, e.g., trypsin, and the reaction surface can also be increased by using beads in microcapillaries or

microfabricated poles in microfluidic devices. After complete digestion, the digested sample is subject to MS/MS or uLC-MS/MS analysis (D->3). (In Fig. 5, (AB) is the immobilized immunoaffinity trapping agent, e.g., anti-mouse IgG heavy chain or Protein G, and (D) is the immobilized trypsin microreactor.

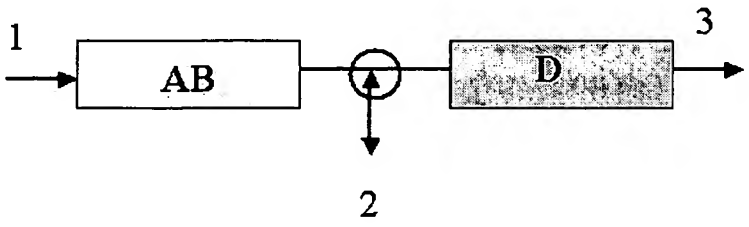

Fig. 4

In high throughput screening mode, the affinity surface of the immunoaffinity trapping chamber is first saturated by the IgG from the hybridoma supernatant (1->AB->2). This step is followed by perfusion with the antigen mixture (1->AB->2). Then, the chamber is washed with PBS (1->AB->2), and the IgG-antigen complex is eluted with an acidic elution buffer into the digestion chamber (D). The pH is adjusted through outlet #2 during the transfer (mixing). After complete digestion, the digested sample is subject to MS/MS or uLC-MS/MS analysis (D->3).

Besides running separate background determination and highthroughput screening steps as described above and de-convolute the results by computer, another strategy can involve separation of the digestion product of the background determination and the screening steps by a serially connected HPLC column. In this latter instance, the non-identical peaks are collected and subjected to MS/MS or uLC-MS/MS analysis.

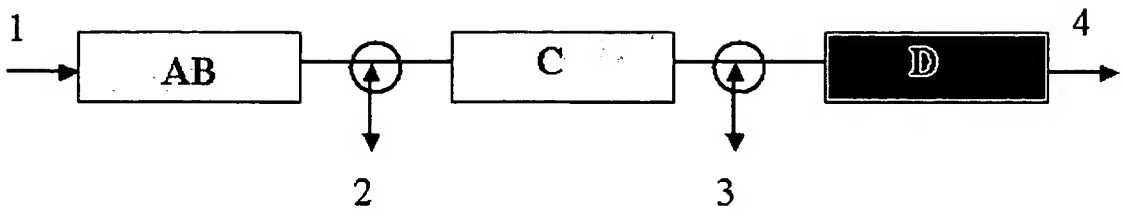

Fig. 5

A different strategy, shown in Fig. 5, would involve HPLC separation between the two chambers, i.e., only the non-identical peaks would be digested and injected to the MS/MS or uLC-MS/MS system. Finally, the system shown in Fig. 4 could include a separation column (C) in between the two chambers (AB) and (D) so that the IgG peak could go to waste through channel #3 and the peak representing the antigen could go into the digestion chamber (C->D) and subsequently to MS/MS or uLC-MS/MS analysis through channel #4.

Alternatively, the same methods can be practiced in a monolithic microfluidics device, either in single channel or parallel channel (96, 384, 1536 lane) structures (Fig. 6).

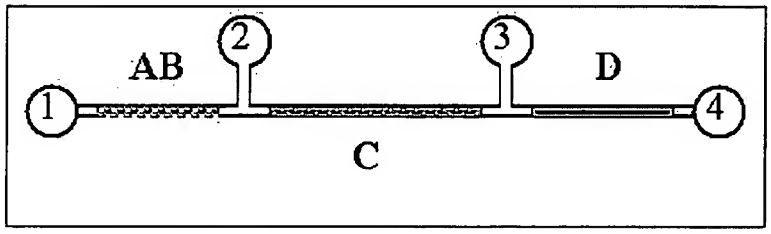

Fig.6

The hybridoma supernatants (mAb precursors) that are determined to have high specificity and good binding properties and that discriminate sets of samples with acceptable confidence levels are selected for individual mAb mediated clinical assay development. The resulting simplex and complex assays are then used for large-scale validation of bio- and diagnostic markers in patient samples in large

cross sectional (validation collection II) and longitudinal clinical studies (e.g., clinical trials.

In another method, large scale protein screening is carried out using nanowell polydimethylsiloxane (PDMS) plates with immobilized linkers in each well (e.g., using avidin, protein A, Protein G, specific anti Ig heavy chain, etc.) capable of high throughput screening of complex analytes, e.g., full plasma proteins or purified disease specific low abundance proteins. The binding assay is accomplished in the nanowells. After all non-specific and unbonded material is washed out, the binding linker is cleaved. The released proteins are digested either *in situ* for nano-ESI/MS (e.g., Nanomate) or transferred to a digestion enzyme-containing membrane that will act as MALDI plate for MS interrogation. To increase precision and detection limit, two or more parallel wells can be used, one for the binding measurement with a reporter only (e.g., fluorescence), the other being used for digestion and MS analysis.

Nano-ELISA: protein A, protein G or gamma Ig is immobilized in the nanowells. During the screening reaction, the immunoglobulin binds to the immobilized linker and then is available to bind the candidate biomarker from the complex analyte sample, e.g., plasma or pooled plasma, "cleared plasma" or pooled "cleared plasma."

A screening assay to Identify hybridoma supernatants that react with pooled complex analyte mixtures uses biotinylated complex analyte, e.g., total plasma proteins or pooled and depleted ("cleared plasma") plasma mixture. If binding is detected, a biomarker hit has been identified.

In semi-quantitative differential screening, two or more samples of complex analyte pools are compared. For example, pooled total plasma protein, or pooled low abundant ("cleared plasma") protein, from a point of care assay and a

control, both biotinylated, could be compared. Detection of the signal intensity difference identifies a biomarker hit.

A quantitative screening assay of individual complex analyte samples builds on the first assay. Non-biotinylated individual complex analyte samples are titrated ad described but this time, only a selected set of hybridoma supernatants are screened, those that were identified as hits in the first assay. Titration curves provide quantitative measure of specific antigen concentration in each individual analyte sample. IC50 values will be used for comparison, and statistical analysis of the entire tested set of individual complex analytes, e.g., a set of 50 disease plasma samples, will be compared to a set of healthy control plasma samples.

Detection can be, e.g., by fluorescence, radioactive, colorimetric, proximity or enzymatic techniques as appropriate. In the example described herein, avidin-biotin-peroxidase (ABC) complexes (Vector) are used to measure binding or binding and competition of biotinylated complex analyte samples, as appropriate.

Parallel microwells or microfabricated microfluidics devices are used for protein ID. Loading of purified protein for ID to MALDI is electronically or manually driven by screening results and performed, e.g., on all hits.

EXAMPLE IV

Antibody library strategy

An alternative strategy for the mAb based biomarker discovery is to use large non-redundant mAb libraries for biomarker discovery and screening via sensitive proteomics chips. In this format, complex analyte mixes are derived from the biological source via enrichment. Hybridoma supernatants are generated and antigen ID is carried out on each mAb containing supernatant. Screening of hybridoma supernatants is stopped when the process saturates and no new

antigen ID's are observed. At this stage, a non-redundant set of mAb's is produced in sufficient quantity for further proteomics chip screening.

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